Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells

Jose B. Cibelli[1,2], Stevem L. Stice[2], Paul J. Golueke[2], Jeff J. Kane[2], Joseph Jerry[1], Cathy Blackwell[2], F. Abel Ponce de León[2,3], and James M. Robl[1,2]*

*[1]Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003. [2]Advanced Cell Technology, Inc., Worcester, MA 01605. [3]Animal Sciences, College of Agricultural, Food and Environmental Sciences, University of Minnesota, St Paul, MN 55108. *Corresponding author (e-mail: robl@vasci.umass.edu).*

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We have developed a method, using nuclear transplantation, to produce transgenic embryonic stem (ES)-like cells from fetal bovine fibroblasts. These cells, when reintroduced into preimplantation embryos, differentiated into derivatives from the three embryonic germ layers, ectoderm, mesoderm, and endoderm, in 5-month-old animals. Six out of seven (86%) calves born were found to be chimeric for at least one tissue. These experiments demonstrate that somatic cells can be genetically modified and then de-differentiated by nuclear transfer into ES-like cells, opening the possibility of using them in differentiation studies and human cell therapy.

Keywords: agricultural biotechnology, nuclear transplantation

Embryonic stem (ES) cells have been available for several strains of mice for many years and have been shown to be capable of contributing to each of the tissues of the animal when combined with a host embryo to form a chimera. Techniques have been developed for inducing the differentiation of mouse ES cells in vitro and successfully transplanting them into recipient mice[1,2]. Success in developing pluripotent cell lines from large animal species, such as bovine, has been minimal. Production of putative bovine ES cells was first reported by Saito et al.[3] and later, a similar type of stem-like cell was reported to direct development through organogenesis[4]. Bovine ES cells that are capable of complete differentiation to term, in vivo, have not been reported. Little success has been achieved in inducing ES cells to differentiate into a specified tissue in vitro or in selecting specific cells, out of the many other types of cells that are present, following the induction of in vitro differentiation.

The objectives of this study were to develop an efficient procedure for producing bovine ES-like cells, to test the pluripotency of these cells in vivo by forming chimeras with host embryos, and to develop an efficient method for genetic modification of the cells using somatic cell nuclear transplantation.

Results

Production of transgenic embryo-derived pluripotent ES-like cell colonies. As one approach to producing transgenic cattle, putative bovine ES-like cells were derived from embryos. In vitro maturation and fertilization of oocytes and in vitro culture of the embryos to the blastocyst stage produced 49 embryos at day 7. Blastocysts were mechanically dissected and plated on mitotically inactivated fetal mouse fibroblast feeder layers. Twenty-seven inner cell masses attached to the feeder layer grew as ES-like cell colonies and successfully survived passaging over at least 12 months without differentiation. These colonies had well-defined edges. Cells in these colonies had a high nuclear to cytoplasmic ratio and a high density of cytoplasmic lipid granules, and were negative for cytokeratin and vimentin. Unlike mouse ES cells, bovine ES cells eventually formed single layer sheets (Fig. 1A) and were alkaline phosphatase negative.

The method of producing transgenic bovine ES-like cells also differed from procedures used for the mouse (Fig. 2A). Bovine ES-like cells, unlike mouse ES cells, do not survive replating when trypsinization is performed; therefore, mechanical passage was used instead. Passage of the cells mechanically involves removing a group of cells, containing a minimum of 50 to 100 cells, and transferring these to fresh feeder layers. Because single cell suspensions could not be passaged, it was not possible to use electroporation for DNA transfection or to clonally propagate transgenic cells. Therefore, microinjection of DNA into the nucleus of individual cells was used as an alternative method. Approximately 500 to 1000 cells could be injected per hour, and injection volume was based on nuclear swelling. Three different cell lines were used. A cytomegalovirus (CMV)-β-galactosidase-neomycin (β-Geo) cassette was delivered into the nucleus of ES-like cells. Five, three, and zero stable, G418 selected transgenic colonies were produced out of 3753, 3508, and 3502 injected cells, respectively. We did not determine if these colonies were derived from single or multiple transgenic cells. During G418 selection the original colony essentially disappeared before growth of the transgenic cells began, indicating a possible clonal origin; however, the possibility of having produced a transgenic colony from two or more closely placed transgenic cells cannot be ruled out. β-galactosidase expression was consistently high in all colonies, although not all cells within a colony expressed the gene (Fig. 1B). PCR amplification of a segment of the transgene also confirmed that the cells were transgenic (Fig. 1E).

Production of transgenic somatic cell-derived bovine pluripotent ES-like cell colonies. Although transgenic ES-like cells can be produced by microinjection, the generation of a large number of

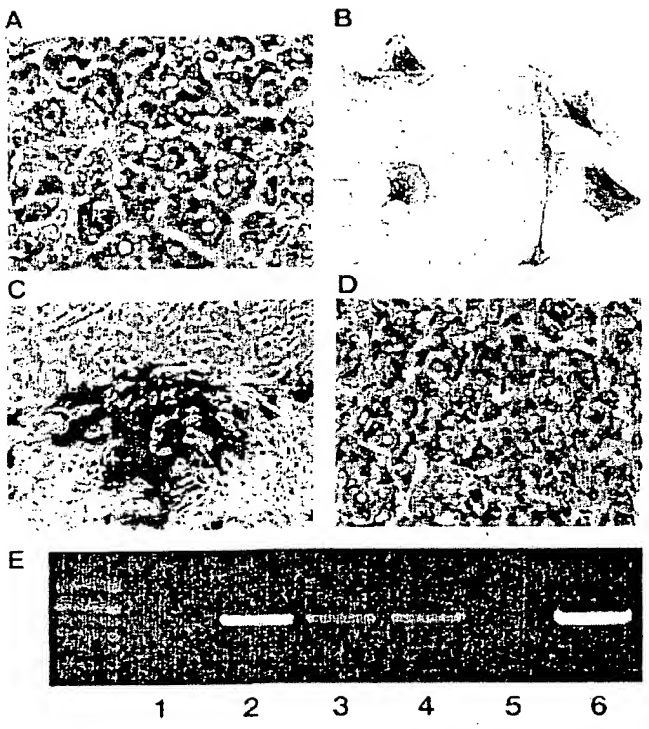

Figure 1. Generation of transgenic ES-like cells. (A) Embryo-derived ES-like cells. (B) β-galactosidase activity of transgenic embryo-derived ES-like cells. (C) β-galactosidase activity of transgenic fetal fibroblasts. (D) Nuclear transfer-derived ES-like cells. (E) PCR ethidium bromide gel of β-galactosidase fragment. Lane 1: nontransgenic embryo-derived ES-like cells; lane 2: transgenic embryo-derived ES-like cells; lane 3: transgenic fetal fibroblasts; lane 4: transgenic nuclear transfer-derived ES-like cells; lane 5: nontransgenic fetal fibroblasts; lane 6: template.

Table 1. Production of transgenic calves using embryo, and NT-derived ES like cells.

	Injected embryos	Blastocyst produced (%)	Blastocyst transferred	Calves born	Transgenic calves*
Embryo ES-like cells	70	16 (23)	16	5	3
NT ES-like cells	99	22 (22)	10	7	6

*Animals with at least one transgenic tissue.

transgenic ES-like cells and clonal propagation was not achieved. Therefore, we took another approach (Fig. 2B) that involved transfection of bovine fetal fibroblasts and fusion of the transgenic fibroblast cells to enucleated oocytes to produce blastocyst stage nuclear transplant embryos. These embryos were then plated on fibroblast feeder layers to produce transgenic ES-like cell colonies. Bovine fibroblasts were obtained from a 55-day fetus, and grown and transfected by electroporation using standard methods (Fig. 1C). Three hundred and thirty enucleated mature bovine oocytes were reconstructed with actively dividing fibroblasts. Thirty-seven (11%) blastocysts (day 7.5) were obtained and ES-like cell lines were established from 22 (59%) of these. Out of 22 cell lines, 21 were positive for the transgene after PCR amplification of the β-galactosidase fragment. The negative ES-like colony could have originated from a neomycin-resistant fibroblast that lost the β-

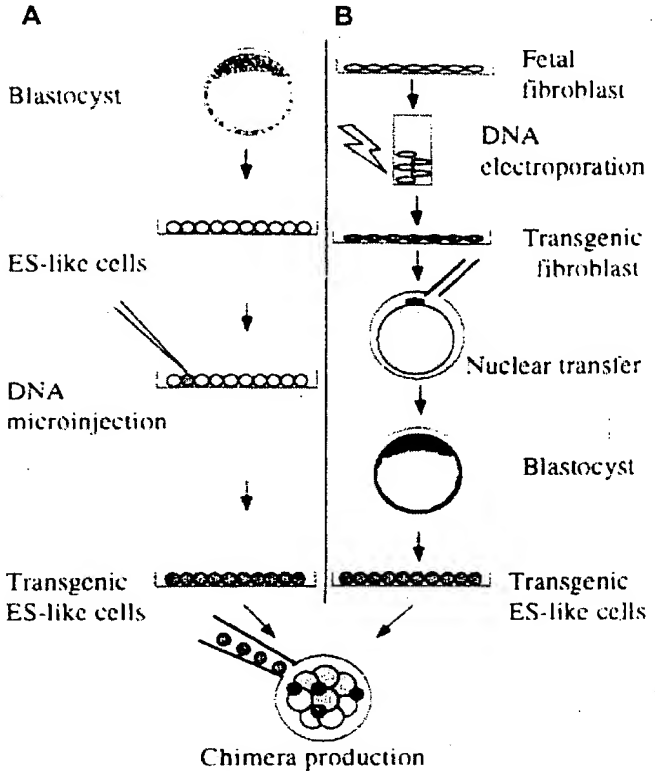

Figure 2. Generation of transgenic ES-like cells (A) by microinjection and (B) by somatic cell nuclear transfer.

galactosidase gene. Fibroblast-derived ES-like cell colonies showed morphology and cytoplasmic marker characteristics identical to those of embryo-derived ES-like cells (Fig. 1D). Furthermore, colonies were passaged for several months without differentiation, even, in one case, when a colony was derived from a senescent, nondividing fibroblast cell line.

Production of chimeric calves. In order to determine the potency of bovine embryo- (passage 10) and fibroblast-derived ES-like cells (passage 3) in vivo, 8 to 10 cells were introduced into day 3 in vitro produced embryos, cultured in vitro until day 7.5 and transferred into synchronized recipients. Five calves were born from embryos that received transgenic embryo-derived ES-like cells, and seven calves were born from embryos that received transgenic nuclear-transfer (NT)-derived ES-like cells (Table 1). All the animals were phenotypically normal.

All the animals were slaughtered at 5 months of age, with the exemption of calf 904, which was killed at 45 days of age. Genomic DNA was isolated from a spectrum of tissues (skin, muscle, brain, liver, spleen, kidney, heart, lung, mammary gland, intestine, and gonads) from each animal, amplified using β-Geo primers, and probed using standard-protocol Southern blot analysis. Results were positive in at least one tissue in nine calves and in two or more tissues in six calves. Oocytes were found to be positive in one animal (Fig. 3). The limited presence of transgenic cells in the newborn animals could be attributed to the fact that not all the ES-like cells were incorporated into the developing morulas; moreover, among those cells that did incorporate, degree of pluripotency may have varied.

Fluorescent in situ hybridization (FISH) analysis was performed in spleen tissue from calf 911 (Fig. 4A), and testis of calf

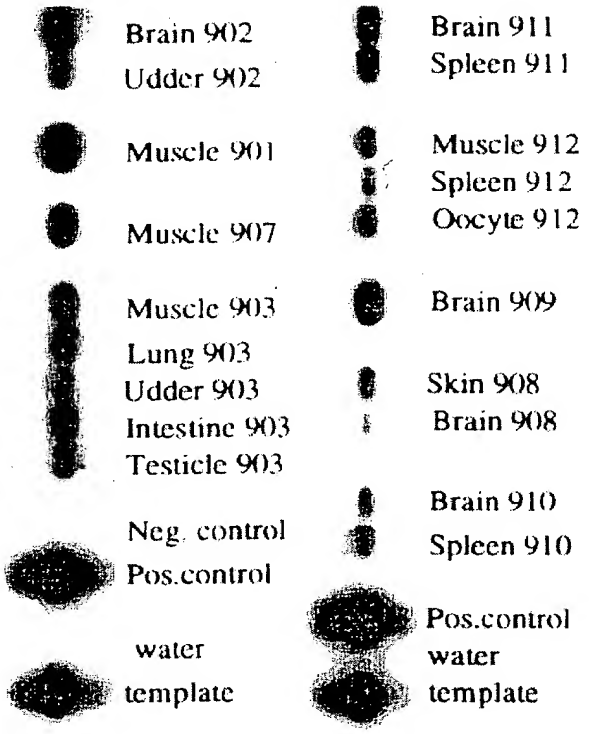

Figure 3. Southern blot analysis of PCR-amplified products of tissues from chimeric calves. Calves 901 to 903 were generated from embryo-derived ES-like transgenic cells. Calves 907 to 912 were generated from nuclear transfer-derived ES-like transgenic cells.

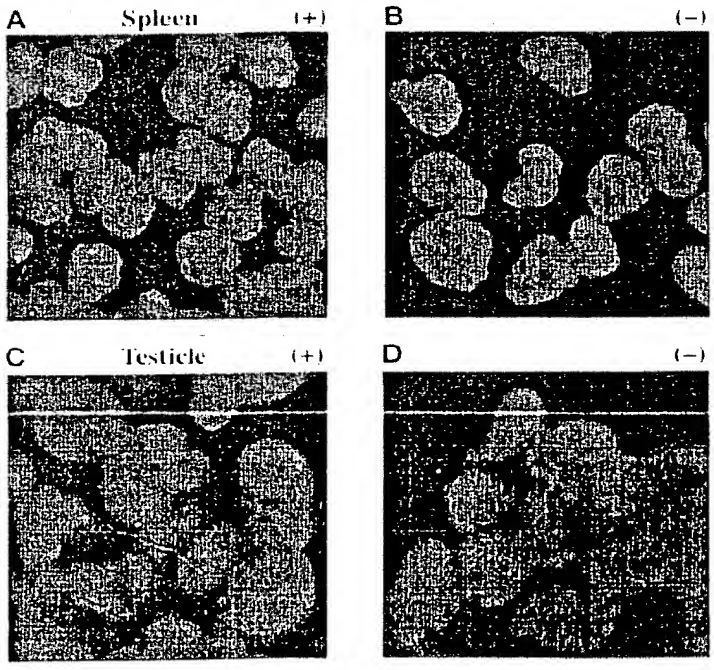

Figure 4. FISH analysis of (A) spleen from calf 911 produced with NT-derived ES-like cells, (B) spleen, negative control, (C) testis of calf 903 produced with embryo-derived ES-like cells, and (D) testis, negative control.

903 (Fig. 4C). Positive hybridization signals were identified in both tissues. In the spleen, 32% of nuclei (82/256) exhibited green signals compared with negative spleen in which only 1% of nuclei (2/231) were classified as carrying green signals. Testis specimens were not presented as a single monolayer of cells; therefore, percentage of positive cells was not assessed; however, positive signals were detected inside the seminiferous tubules.

Discussion

The first objective of this study was to produce bovine pluripotent ES-like cells. ES-like cells are derived from an early stage embryo or the inner cell mass (ICM) directly, and, therefore, should retain the morphology and cellular characteristics of the ICM. In the mouse, ES cells grow as colonies with a defined margin, and cells have high nuclear to cytoplasmic ratio and high density of lipid inclusions similar to the ICM. Our bovine cells derived both from embryos and NT fibroblasts, also retained these characteristics. The expression of various cytoplasmic markers has also been used to indicate an ICM-like quality of mouse ES cells. In the bovine, ES-like cells derived either from embryonic or somatic cell sources, do not express differentiation markers such as vimentin and cytokeratin in a pattern similar to the ICM; however, these cells are alkaline phosphatase negative. The second characteristic of a pluripotent embryonic cell is that it can be grown over many passages without showing signs of differentiation. In this study, and other preliminary work[5], bovine ICM-derived cells were passaged for over 1 year without losing the morphological and cellular similarities to the ICM. The third and most important characteristic used to define ES-like cells is that, upon introduction into a preimplantation embryo, they are able to colonize the ectodermal, mesodermal, and endodermal tissues and the germ line, as the host embryo develops and differentiates. In this study it was shown that both embryonic and fibroblast-derived ES-like bovine cells are capable of giving rise to multiple tissues in 5-month-old animals. Our results demonstrate that cells derived from somatic and embryonic sources possess functional and phenotypic characteristics of pluripotent ES-like cells.

Much work has been done in many different species toward developing methods of producing ES cells; however, little success has been reported at meeting all the criteria listed above. In rabbit[6], production of chimeric offspring was reported, but no chimerism in gonads was demonstrated. In hamster[7,8] and cow[9,4], cells were grown in vitro; however, no chimeric animals were produced. This is the first published report demonstrating transgenic chimerism in full-term live mammals, including in gonadal tissue from a species other than the mouse. However, until germline transmission is demonstrated, we refer to our cells as "pluripotent or ES-like cells" instead of ES cells.

The results in this study indicate that, although genetic modifications could be made directly in bovine ES-like cells by microinjection, and transgenic cells could be selected by a standard neomycin resistance approach, limitations in the number of cells that can be microinjected, the slow growth of the cells, and our inability to clonally propagate the ES-like cells limits the usefulness of this approach, particularly for gene targeting. This is one important difference between bovine ES-like cells and mouse ES cells. Aside from the fact that care must be taken to prevent differentiation, mouse ES cells can be readily grown in culture, clonally propagated, transfected by standard high-volume gene transfer methods, and in many cases, exhibit high-efficiency homologous recombination. In our system, the low transfection efficiency of bovine ES-like cells prevents the possibility of using direct ES-like cell transfection for gene targeting.

An alternative method of making genetic modifications in bovine ES-like cells is to genetically modify fibroblast cells and then produce embryos by nuclear transplantation. Genetic modification is relatively simple with fibroblast cells, which are easy to grow, transfect, and clonally propagate. Furthermore, gene targeting and selection for homozygous lines in vitro have been successful in human fibroblast lines[9].

This study demonstrates that ES-like cells can be produced from bovine embryos, which can be cultured without a change in morphology for indefinite periods in vitro and retain the ability to give rise to tissues derived from all three germ layers in offspring. Furthermore, using nuclear transplantation, these cells can be produced from genetically modified fibroblasts. This system could be useful for the in vitro production of genetically modified bovine cells to be used for cell transplant therapies for many different human diseases.

Experimental protocol

In vitro maturation of bovine oocytes. Ovaries were recovered at a slaughterhouse, placed in warm phosphate-buffered saline (PBS) (34°C) and brought to the laboratory within a limit of 8 h. Each follicle of more than 2 mm in diameter was aseptically aspirated with an 18 gauge needle. Search of oocytes was performed in modified Tyrode's medium (TL Hepes). Oocytes with a homogeneous cytoplasm, considerable perivitelline space and intact cumulus cells were placed in maturation medium M199 (GIBCO, Grand Island, NY), 10% fetal calf serum (FCS), 5 µl/ml bovine follicle-stimulating hormone (Nobl, Sioux Center, IA), 5 µl/ml bovine luteinizing hormone (Nobl), and 10 µl/ml Pen-strep (Sigma, St. Louis, MO) for 22 h at 38.5°C and 5% CO_2.

In vitro fertilization of bovine oocytes. Twenty-two hours postmaturation, oocytes were placed in fertilization medium (5 ml CR2- Specialty medium, stock solution 100 U/ml penicillin, 100 µg/ml streptomycin, 0.005 µg/ml phenol red, 30 mg bovine serum albumin fatty acid free, 5 µg/ml sodium heparin). A unit of frozen semen was thawed and placed on top of a percoll layer that contains 90% percoll (Sigma) and one part 10× modified sperm TL plus, 45% percoll (one part of 90% percoll stock solution and one part sperm TL without BSA). Dead sperm were separated from live sperm by centrifugation at 700 G for 30 min. Sperm pellet was resuspended at a final concentration of 500,000 sperm/ml. After 12 h in culture at 38.5°C and 5% CO_2, eggs were removed and placed in CR2 medium with 3 mg/ml BSA.

Embryo culture. During the first 3 days after fertilization, embryos were cultured in 500 µl well plates with mouse embryonic fibroblast (MF) feeder layers and CR2 with 6 mg/ml BSA. On day 4, embryos were transferred to 500 µl well plates with MF feeder layers, CR2 with 6 mg/ml BSA, and 10% FCS until blastocyst stage (day 7 postinsemination).

ES-like cell culture. Blastocysts were placed in a 32 mm plate (Nunc, Rochester, NY) with mitotically inactivated MF feeder layer and ES medium (Alpha MEM, 10% fetal calf serum, 4 µl/ml antibiotic-antimycotic, 2.8 µl/ml 2-mercaptoethanol, 0.3 mg/ml L-glutamine, and 1 µl/ml tylosin tartrate) equilibrated a day in advance at 38.5°C and 5% CO_2. Using a 22 gauge needle, blastocysts' zona pellucida and trophoblast were mechanically removed. The remaining ICM was placed underneath the MF. After 1 week in culture, ES-like cells were passaged to a fresh mitotically inactivated MF. Inactivation of MF was performed by exposing them to gamma radiation (2956 rads). ES-like cells were passaged by cutting a small piece (50 to 100 cells) of the colony and placed on top MF feeder layers using a pulled Pasteur pipette.

Nuclear transplantation. Eighteen hours postmaturation, oocytes were placed in a 100 µl drop of TL HECM-Hepes under mineral oil (Sigma). Oocyte enucleation (extraction of chromosomes) was performed using a beveled glass pipette of 25 µm diameter. Evaluation of enucleation was done by exposure of individual oocytes previously cultured for 15 min in 1 µg/ml of bisBENZIMIDE (Hoechst 33342; Sigma) in TL HECM-Hepes under ultraviolet light. Donor cells were placed in the perivitelline space and fused with the egg's cytoplasm at 23 h postmaturation. Oocytes and donor cell were placed into 4 ml medium made of 50% SOR2 fusion medium (0.25 M D-sorbitol (Sigma), 100 (M CaOAc (Sigma), 0.5 mM magnesium acetate (Sigma), 1.0 g BSA (Sigma), and 50% HECM-Hepes for 2 min. Eggs were then placed between the electrodes of a 500 µm fusion chamber. Once the eggs were aligned, a pulse of 90 V was administered over 15 µs. Eggs were then returned to the 50/50 medium of SOR2 and HECM/Hepes for 2 min and, finally, placed into a 500 µl drop of CR2 at 38.5°C and 5% CO, until activation.

Oocyte activation. Activation was performed in general as described[11]. Briefly, 25 to 27 h postmaturation oocytes were incubated in 5 µm ionomycin (Cal Biochem, La Jolla, CA), and 2 mM of 6-dimethylaminopurine (DMAP; Sigma) in CR2 with 3 mg/ml of BSA (fatty acid free; Sigma). After activation, eggs were washed in HECM/Hepes five times and placed for culture in a 500 µl well of MF and CR2 with 3 mg/ml of BSA (fatty acid free) at 38.5°C and 5% CO_2.

Transgenic ES-like cell production. Five micrograms per milliliter of a β-Geo cassette gene were microinjected into the nuclei of bovine ES-like cells. Twenty four to forty-eight hours after microinjection, 150 µg/ml of G418 was added to the culture medium. After 3 weeks under selection, a colony was considered transgenic upon DNA screening by PCR and ethidium bromide gel, and by β-galactosidase staining.

Bovine fibroblast production and electroporation. Bovine fibroblasts were produced from a 55-day-old fetus as follows. Under sterile conditions, the livers, intestines, and heads of the fetuses were discarded. The remaining parts of the fetuses were carefully minced and placed in a solution of Delbucco's phosphate buffered saline (DPBS) with 0.08% trypsin (Difco, Detroit, MI) and 0.02% EDTA (Sigma). After 30 min incubation at 37°C the supernatant was discarded and the pellet resuspended with trypsin-EDTA/DPBS. After 30 min incubation, the supernatant was removed and centrifuged at 300 G for 10 min. The pellet of cells was then resuspended with ES culture medium and plated in polystyrene tissue culture dishes (25010; Corning, Charlotte, NC). After two passages, cells were electroporated with a β-Geo cassette gene with the protocol described by Invitrogen (San Diego, CA) for COS cells[11]. After 3 weeks under 400 µg per ml of G418 selection, fibroblasts were considered transgenic upon DNA screening by PCR and ethidium bromide gel, and by β-galactosidase staining.

Alkaline phosphatase staining. Culture medium was removed from the plates and cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed three times in Tris-maleate buffer (3.6 g Trizma base [Sigma], in 1 L water, pH raised to 9.0 with 1 M maleic acid) for 10 min each wash. The last wash was removed and the staining solution (Tris-maleate buffer, 200 µl of a 0.5 mM $MgCl_2$, naphthol AS-MX phosphate [Sigma], 0.4 mg/ml, Fast blue [Sigma], 1 mg/ml) was added to the cells for 15 to 20 min. Once blue cells were detected, the reaction was stopped by adding PBS which brought the pH to 7.4.

Chimera production. Seventy-two hours after in vitro fertilization (eight cell stage), embryos were placed in manipulation medium (HECM/Hepes with 10% FCS and 7.5 µg/ml of cytochalasin B [Sigma]). ES-like cells were dissociated using 0.08 % trypsin (Difco) and 0.02% EDTA in PBS during 25 to 30 min. Using a 15–20 µm diameter beveled pipette, 8 to 10 cells were introduced into the embryos. Embryos were placed in a 500 µl culture drop (MF feeder layer, CR2 with 6 mg/ml of BSA and 10% FCS).

Immunohistochemical studies. Primary antibodies specific against cytokeratin 8–18 (Sigma) and vimentin (Sigma) were used in ES-like cell cultures. Cells were plated on sterile glass slides, fixed in 2% paraformaldehyde, and extracted with cold (-20°C) acetone. Cells were incubated with primary antibody dilutions in PBS containing 0.5% BSA (PBSA) for 1 h at room temperature. Slides were then rinsed three times in PBSA with changes of rinse solution every 10 min, and incubated for 1 h in fluorescein 5-isothiocyanate (FITC) conjugated antimouse IgG (Sigma). After rinsing in PBSA for 30 min, coverslips were mounted in 50% glycerol and observed under a fluorescence microscope[9].

β-galactosidase staining. Culture medium was removed from the plates, and cells were fixed with 2% glutaraldehyde in PBS. Then cells were washed three times with PBS and color substrate (5 mM $K_3Fe(CN)6$, 5 mM $K_4Fe(CN)6$, 1 mM $MgCl_2$, 1 mg/ml X-gal in PBS, pH 7.0–7.5) was added for 3 h[11].

PCR analysis and blot analysis. Analysis of transfected cells and tissue from 5-month-old animals was performed using a sense primer (ACT3βGeo, a 21 base CGCTGTGGTACACGCTGTGCG) and antisense primer (ACT4βGEO, a 22 base CACCATCCAGTGCAGGAGCTCG [Amitof Biotech, Boston, MA). Reactions were run for 35 cycles (1) heated at 95°C for 30 s (2) primers were annealed at 65°C for 1 min, (3) extended for 2 min at 72°C, followed by 10 min extension at 72°C. The amplified product was a 782 bp fragment. Sample analysis was performed by separating by size in a (1%) TAE agarose gel electrophoresis containing ethidium bromide. Products were sized by comparison with markers consisting of 1444 bp, 943 bp, 754 bp, 585 bp, 458 bp, 341 bp, 258 bp, 153 bp, and 105 bp. DNA was then handled according to standard Southern blot analysis protocols. Briefly, DNA was transferred to Zetabind (Cuno, Meriden, CT) by capillary transfer and

probed with a gel-purified 289 bp ClaI to EcoRV fragment labeled with ^{32}PdCTP using random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was done at 42°C overnight. After washing, the blot was exposed to Biomax film (Kodak, Rochester, NY) overnight. Nontransgenic fibroblasts and water were used as negative controls, and transgenic cells for β-Geo and template were used as positive control. When oocytes were analyzed, ovarian follicles were aspirated with a syringe using an 18 gauge needle. Eggs' granulosa cells were removed by vortexing the oocyte/cumulus cell complex in 5 mg/ml of hyaluronidase (Sigma) in PBS. Oocytes were washed five times in PBS before DNA isolation.

FISH analysis. Samples were frozen and made onto slides either by slightly pressing the sample against the slide (for spleen slides) or by cryosections (for testis slides). β-Geo DNA was linearized with ScaI and biotin-labeled by nick translation reaction. An aliquot of the biotin-labeled DNA was run on a gel and transferred to a membrane, and a streptavidin-alkaline phosphatase assay was performed to detect the size of labeled fragments and quantity of biotin incorporation. The labeled DNA was then coprecipitated with salmon sperm DNA as carrier. A number of single-target single-color FISH assays were performed using varied concentrations of labeled DNA as a probe (250–500 ng). The specimens were washed in 70% acetic acid and digested in pepsin (0.01% in 0.01M HCl at 37°C) before denaturation. Testis slides were incubated in pepsin at room temperature for 10 min before warming to 37°C. Denaturation was performed at 75°C for both chromosomal and probe DNAs and hybridization was allowed to occur for approximately 60 h. Posthybridization washes included three 5 min washes in 50% formamide/2XSSC and three 5 min washes in 2XSSC at 43°C. Immunochemical detection was achieved with consecutive incubations in FITC-avidin, biotinylated anti-avidin and FITC avidin (Vector, Burlingame, VT). Chromatin was counterstained with DAPI (0.01 μg/ml on antifade; Boehringer Mannheim). After hybridization, slides were coded and blindly analyzed. Analysis was performed in an Olympus BX-60 fluorescence microscope using interference filter sets for single band (DAPI and FITC) and triple band (DAPI, FITC, Texas red). Gray images were acquired using a CCD camera (Photometrics, Phoenix, AZ) and combined using the Oncor (Gaithersburg, MD) image software.

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